

Myogenic Differentiation of Human Adipose-Derived Stem Cells

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Objective Cell therapy has been extensively studied as a gene complementation approach in muscular dystrophy including Duchenne muscular dystrophy (DMD), and adipose tissue has recently been identified as a uniquely abundant and adequately accessible source of pluripotent cells. In the present work, we investigated myogenic potentials of adipose-derived stem cells (ADSCs) depending on culture media and isolation with using surface markers.

Method Human ADSCs were obtained by liposuction and cultured in two different media; control and myogenic media. In addition we attempted to isolate ADSCs by utilizing surface markers: CD45 and CD133. The following observations were made to evaluate myogenic differentiation as the expression of myogenic regulatory factors (MyoD, Myf-5 and Myf-6) and desmin by RT-PCR and immunofluorescence study.

Results Conversion of ADSCs to myogenic phenotype was observed by indirect immunofluorescence study of MyoD and Myf-5 in regardless of media type and isolation method. In addition mRNA of MyoD and Myf-5 were positive in both culture media, and there were no differences of MyoD and Myf-5 responses between CD45- and CD45-CD133-ADSCs. However, secondary myogenic regulatory factor (Myf-6) was not expressed constantly, and desmin were negative in all cultural condition.

Conclusion Our findings suggest that human ADSCs might have myogenic potentials. However, further studies are needed to express the secondary myogenic regulatory factors and proteins in myoblasts.

Key Words Muscular dystrophy, Stem cell, Differentiation, Myogenic regulatory factor, Desmin

INTRODUCTION

Duchenne muscular dystrophy is recessively inherited through sex chromosomes. It is known to be caused by the genetic variation of dystrophin located in the short arm (Xp21) of the X chromosome, and occurs in 1 out

of 3,500 baby boys.¹⁻³ There is currently no cure for DMD, although comprehensive rehabilitation inducing physiotherapy, kinesiotherapy and pulmonary rehabilitation have been adhibited to minimize progress and complications.² But in recent times, intensive research is underway. One of those experimental treatments is

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based on myoblast transfer. The myoblast transfer has been developed to fuse normal cells with the patients' myocytes and thus to induce the production of dystrophin protein. However, this approach has a number of limitations including a low rate of spread, the poor survival of the injected myoblasts, and an insufficiency of myoblasts, so there has been still controversy as to its clinical efficacy.³⁻⁶

In particular, immunorejection is a principal problem in myoblast graft. To solve the problem, an attempt has been made to apply stem cells obtained from cord blood, bone marrow, muscles and fat. Adipose tissue eases donors' burden as it can be collected through liposuction, as well as tissue can be collected in quantity.⁷⁻¹² However, the problem is that adipose-derived stem cells (ADSCs) may be differentiated into various tissues such as bone, cartilage and muscle as most of them are mesenchymal tissues. Thus, it is imperative to control them to be differentiated only into myocytes in the process of isolation and culture.¹³

Embryologically, it is known that various factors are concerned in the process of myogenic differentiation, but the whole process has not been unraveled yet. Meanwhile, the basic helix-loop-helix (bHLH) transcription factor, *i.e.*, a myogenic regulatory factor (MRF), has been reported to take an important role. MyoD and Myf-5, primary MRFs, lead ADSCs to myoblasts. Myogenin and Myf-6, secondary MRFs, differentiate myoblasts into myotubes,¹³⁻¹⁵ which can be proved by morphologic changes, caused by stem cells' being differentiated into myocytes, and MRFs' being expressed in the process of differentiation.

Since it is difficult to control the differentiation of stem cells, various differentiations have been attempted under various conditions. The diversification of media is one of such methods.^{12,13,16,17} But according to previous studies, the diversification of media has limitations to the perfect myogenic differentiation. To overcome such limitations, it may be effective to separately culture myogenic stem cells. In this connection, surface markers, such as CD45, CD133, CD34, Sca-1 and c-kit, can be used to sort cells effective for myogenic differentiation.^{18,19}

The Purpose of this study was to identify proper conditions for myogenic differentiation, by extension, to ascertain whether surface markers are useful to sort cells. In line with these objectives, an observation was made of whether myogenic differentiation would be influenced by different media of which composition was

adjusted by stem cells, for which the expression of MRFs was checked. In addition, an observation was made of the differentiation of adipose-derived stem cells sorted by surface markers.

MATERIALS AND METHODS

Cell sorting and primary culture

HURIM BIOCELL, a biotech company located in Seoul, Korea, primarily cultured ADSCs by using adipose tissue collected through liposuction, and delivered them to researchers as in the following.

The collected adipose tissue was rinsed with phosphate buffered saline (PBS, GIBCO BRL, Grand Island, USA) 2 times, which removed blood and foreign substances. Then, it was suspended in 0.075% collagenase (Sigma, St. Louis, USA) which dissolved in PBS at 37°C for 30 minutes, whereby extracellular matrix was lysed. For reference, the mixture was the same amount as the adipose tissue. After that, it was mixed with Dulbecco's modified Eagles medium (DMEM, GIBCO) and 10% fetal bovine serum (FBS, GIBCO) in order to decelerate lysis. Next, it was centrifuged at 250 g for 10 minutes, which precipitated cells. The precipitated cells were treated with culture medium (10% FBS-DMEM) and 0.16 M NH₄Cl, waited for 10 minutes in room temperature, and then were centrifuged at 250 g for 10 minutes. The precipitate was soaked in culture medium (10% FBS-DMEM), and was divided onto 75-square-centimeter culture flasks (NUNC, Rocklde, Denmark) by 1×10⁶/mL. It was cultured in the 5% CO₂ incubator at 37°C, and the culture medium was replaced at intervals of 1 week. When the confluence of cells reached 70% and over, cells were separated from the culture dish by the use of 0.05% trypsin-0.53 mM EDTA. After being centrifuged, it was subcultured. Since then, the culture medium was replaced at intervals of 3 days.

Induction of myogenic differentiation

Media-based differentiation: After being primarily cultured, cells were divided into two groups. One group was cultured in a medium composed of 10% FBS-DMEM, 1% penicillin and streptomycin (GIBCO). The medium was defined as the control medium, and the group cultured in the control medium was defined as the control group. The other group was cultured in a medium composed of the control medium, 5% horse serum (GIBCO) and 50 μM hydrocortisone (Sigma). The

medium was used in previous studies.^{12,20} The medium was defined as the myogenic medium, and the group cultured in the myogenic medium was defined as the myogenic group. The two groups were cultured in the 5% CO₂ incubator at 37°C for 21 days, whereat the expression of MRFs and myofascial protein, *i.e.*, desmin, was observed through immunofluorescence stain and Western blot. The expression of mRNA was observed through RT-PCR.

Surface markers-based differentiation: CD45 and CD133, markers of myogenic stem cells, were applied to magnetic-activated cell sorting (MACS) as in the following:

After being primarily cultured, ADSCs were treated with the CD45 antibody (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) in the proportion of 1 : 100 at room temperature for 30 minutes. Cells, which responded to the primary antibody, were cleansed with 1×PBS (original concentration) 2 times. Then, they were made to respond to 20 µL of goat anti-mouse IgG microbeads (Miltenyi Biotec GmbH) at 6 to 12°C for 15 minutes after being soaked in 80 µL of PBS. Next, the supernatant was removed by centrifugation after dilution with decuple PBS. By the use of MiniMACS separation system (Miltenyi Biotec GmbH), the cells were divided into ones sorted by the magnetic field (CD45+) and ones were not sorted (CD45-), only CD45- cells of which were cultured. Likewise, CD133 cells were sorted by the use of CD45- cells in the same way. A group composed of cells that responded negatively to CD45- cells and another group of cells that responded negatively not even to the CD133 antibody (Miltenyi Biotec GmbH) (CD45-CD133-) were cultured in the control medium and the myogenic medium respectively. An analysis was made of differentiation, like in primarily cultured cells.

Analysis of differentiation

RT-PCR (reverse transcription-polymerase chain reaction): The differentiated cells were washed with 1×PBS 2 times. Total RNAs were extracted with the Trizol Reagent as per the manufacturer's protocol (Invitrogen, Carlsbad, USA). Three microgram amounts of total RNA were reversetranscribed using M-MuLV reverse transcriptase (Promega, Madison, USA) at 37°C for 1 h. Gene expression of human cells was evaluated using primers specific for human cDNAs. These were implemented by the use of PTC-500 thermocycler (MJ Research, Waltham, USA). The followings are primer pairs used for amplification.

MyoD forward: 5'-GTCGAGCCTAGACTGCCTGT-3', reverse: 5'-GGTATATCGGGTTGGGGTTC-3'

Myf-5 forward: 5'-GCCTGAAGAAGGTCAACCAG-3', reverse: 5'-CCATCAGAGCAGTTGGAGGT-3'

Myf-6 forward: 5'-GCCAAGTGTTTCCGATCATT-3', reverse: 5'-CTTCTCCACCACTTCCTCCA-3'

Desmin forward: 5'-CCTACTCTGCCCTCAACTTC-3', reverse: 5'-AGTATCCCAACACCCTGCTC-3'

Immunofluorescence stain: The cultured cells were washed with 1×PBS after being fixed with a mixture of cold acetone and methanol (1 : 1). After this, the samples were blocked with PBS containing 2% bovine-serum albumin (BSA, Sigma) and 5% goat serum (Sigma) for 30 minutes at room temperature, and incubated with the primary antibody (Santa Cruz Biotechnology, Santa Cruz, USA) of MyoD, Myf-5, Myf-6 and desmin overnight at 4°C. The cells were washed with 1×PBS 3 times and then they were kept at room temperature for 30 minutes with secondary species-specific Abs (Vector Laboratories, Inc., USA). They were washed with 1×PBS 3 times, incubated with avidin-fluorescein isothiocyanate conjugated (FITC) for 30 minutes at room temperature, and again washed with 1×PBS. The ensuing color development was observed through a fluorescence microscope (Nikon Eclipse 80i, Nikon, Tokyo, Japan). MyoD, Myf-5 and Myf-6 were regarded as positive only when their nuclei were stained. In the case of desmin, the positive reaction was confined to cases where cytoplasm was stained.

RESULTS

Differentiation in primarily cultured cells

Expression of MRFs (MyoD, Myf-5 and Myf-6): A 28-day culture was performed with the control medium and the myogenic medium, during which the mRNA levels of MRFs (MyoD, Myf-5 and Myf-6) were measured by RT-PCR. MyoD was expressed in both groups. In the case of the control group, the expression of MyoD increased in proportion to time. In the myogenic group, expression increased immediately after replacement of medium but gradually decreased with time. Myf-5 was similar to MyoD in the pattern of expression, but its expression more increased in the control group than in the myogenic group. The expression of Myf-6 was more increased in the myogenic group than in the control group, but its increase was gradual, unlike MyoD and Myf-5 (Fig. 1).

To examine the protein expression changes of MRFs under different conditions, primary cells were cultured for 7 and 21 days respectively using control or myogenic medium. In the case of MyoD and Myf-5, intranuclear expression increased 7 days later in both the control medium and the myogenic medium, but intergroup significant differences were not observed. Myf-6 did not show intranuclear staining even 21 days later (Fig. 2).

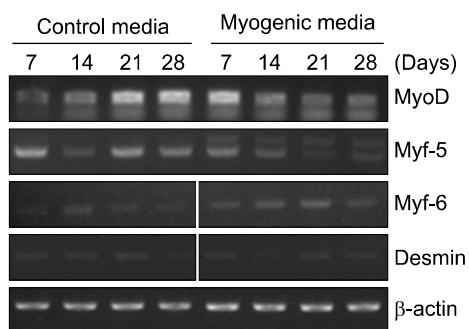


Fig. 1. Myogenic differentiation of primary cultured ADSCs (adipose-derived stem cells), as determined by RT-PCR for expression of MyoD, Myf-5, Myf-6 and Desmin mRNAs. Expression of β -actin mRNA was used as a loading control. ADSCs were cultured in each control and myogenic media.

Desmin: During the period, any significant expressions were not observed not only in protein but in mRNA regardless of the conditions of media (Fig. 1 and 2).

Differentiation in surface markers

Expression of MRFs (MyoD, Myf-5 and Myf-6): ADSCs, divided into CD45⁻ and CD45⁻CD133⁻, were cultured in the control medium and the myogenic medium respectively for 49 days, during which the mRNA expressions of MyoD, Myf-5 and Myf-6 were measured by RT-PCR at intervals of 2 weeks. MyoD was expressed throughout the period and seemed to increase with time, but did not show a meaningful tendency. Myf-5 was more expressed in CD45⁻ and CD45⁻CD133⁻ control media than in the myogenic medium. The expression of Myf-6 was not observed throughout the period (Fig. 3).

Cells, divided into CD45⁻ and CD45⁻CD133⁻, were cultured in the control medium and the myogenic medium for 21 days. On the immunofluorescence stain test, MyoD and Myf-5 were observed in both groups, but intergroup significant differences were not observed. Myf-6 did not show intranuclear staining in both groups.

Desmin: During the study, protein was not expressed not only on the immunofluorescence stain test but on

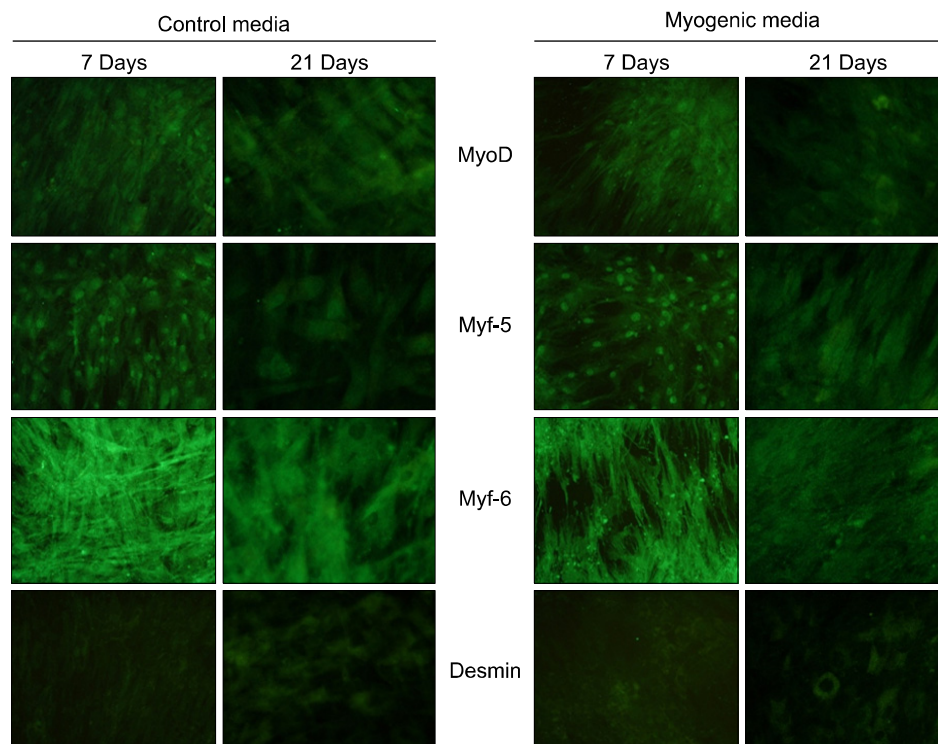


Fig. 2. Myogenic regulatory factors and desmin expression by ADSCs (adipose-derived stem cells) cultured in control and myogenic media. ADSCs were stained with avidin-FITC (fluorescein isothiocyanate conjugated) and visualized by a fluorescent microscope on days 7 and 21. By day 7 MyoD and Myf-5 were extensively expressed in the nuclei of most cells ($\times 200$). However, Myf-6 was not expressed in the nuclei, and desmin was totally negative through day 21.

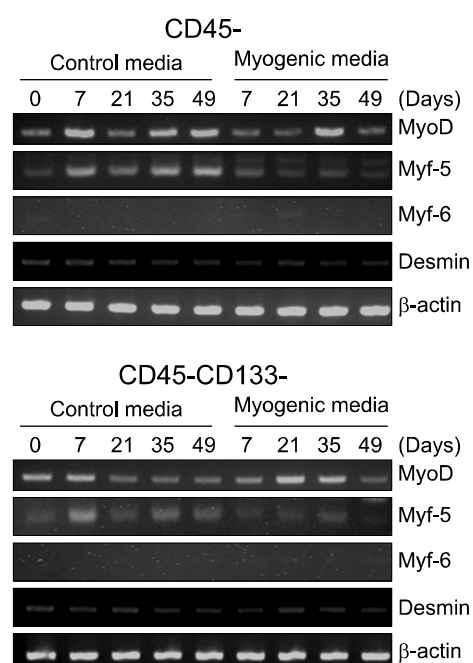


Fig. 3. Myogenic differentiation of isolated ADSCs with using CD45/CD133, as determined by RT-PCR for expression of MyoD, Myf-5, Myf-6 and Desmin mRNAs. Expression of β -actin mRNA was used as a loading control. ADSCs were cultured for 42 days in each control and myogenic media.

the Western blot regardless of surface markers (not shown), and besides, significant changes were not observed even in the mRNA level (Fig. 3).

DISCUSSION

Mammals' adipose tissue consists of adipocytes and collagenous fiber in addition to the stromal vascular fraction. Thus, it has been reported that adipose tissue can be variously differentiated according to culture conditions, in common with bone marrow.^{12,21,22} Asakura et al.²³ and Csete et al.²⁴ reported that myoblasts could be differentiated into osteocytes and adipocytes, which shows the possibility that musculoskeletal cells, osteocytes, chondrocytes and adipocytes have the same mesenchymal origin and they share regulatory process and factors when they are differentiated into each organ. Mesenchymal stem cells, extracted from such organs, are easily differentiated into related cells.²⁵

So far, mesenchymal stem cells have been extracted from bone marrow in many cases. But it causes inconvenience to donors and also they cannot be produced

in quantity. As the solution, interest has been concentrated on adipose tissue extracted through liposuction.

Several studies reported that ADSCs could be differentiated to hoped-for cells by changing the compositions of media.^{12,17,22} In this study, the expression of primary MRFs showed a positive reaction with time, regardless of the conditions of media. But when the differentiation of primarily cultured cells was induced the myogenic medium, MyoD was early expressed and decreased as compared to the control medium, which is assumed that the myogenic medium would accelerate the myogenic differentiation of ADSCs. It is because in the process of myogenic differentiation, secondary MRFs are expressed after primary MRFs are activated, and then primary MRFs tend to be deactivated. But in the process where myogenic differentiation, MyoD and Myf-5, primary MRFs, showed positive reactions on the immunofluorescence stain test early in culture. On the other hand, Myf-6, a secondary MRF, showed a negative reaction even after culture. In the case of desimin protein in myoblasts, a negative reaction was continuously maintained and as a result was judged to be unsuitable for myogenic differentiation. The results imply that a perfect differentiation cannot be induced by myogenic medium used in this study and the applied condition. Accordingly, there is a need to conduct a further study with factors to promote differentiation and various analyses.

ADSCs, cultured after being sorted by CD45 and CD133, were not much different from primarily cultured cells in differentiation. It means that cells, sorted by surface markers, are not effective to induce differentiation. Thus, it may be effective to apply other methods to the induction of differentiation without additional cell sorting.

CONCLUSION

In this study, a myogenic differentiation was partially induced by the use of ADSCs. These findings suggest that human ADSCs might have myogenic potentials. So the results of this study are expected to be a basic step for the differentiation of ADSDs into myoblasts. However, further studies are needed to express the secondary myogenic regulatory factors and proteins in myoblasts.

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